Chromosomal β -lactamase expression and antibiotic resistance in *Enterobacter cloacae*

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Summary. The activities of β -lactam antibiotics were compared against *Enterobacter cloacae* clinical isolates and mutants which had inducible, stably-derepressed, and basal expression of a *pI* 8.4 subtype of the Ia chromosomal β -lactamase. These activities were correlated with the results of studies of the β -lactamase-lability and β -lactamase-inducer-power of the antibiotics. Cefoxitin and ampicillin were labile, and induced β -lactamase production strongly at concentrations below their MIC values. Consequently, β -lactamase-inducible and β -lactamase-stably-derepressed organisms were highly resistant (MIC > 256 mg/L) to these antibiotics, whereas enzyme-basal strains and mutants were much more susceptible (MIC 1–16 mg/L). Imipenem also induced β -lactamase production strongly at concentrations below its MIC, but was more stable than ampicillin and cefoxitin. It was active against enzyme-inducible and stably-derepressed organisms at 0.25–0.5 mg/L and against β -lactamase-basal organisms at 0.06–0.25 mg/L. Thus the β -lactamase afforded only very low-level protection against imipenem; this appeared to be by a non-hydrolytic mechanism, with the enzyme binding to the antibiotic in a relatively stable complex. This complex, which probably was an intermediate in a hydrolytic pathway, was isolated by gel-filtration chromatography and shown to have a breakdown half-life of 47 ± 2 min. Cefotaxime, ceftriaxone and mezlocillin were labile to the *pI* 8.4 β -lactamase but induced β -lactamase production weakly at concentrations below their MIC values. Consequently, β -lactamase-inducible and β -lactamase-basal organisms remained equally susceptible (MIC 0.06–4 mg/L), but stably-derepressed organisms were considerably more resistant (MIC > 64 mg/L) to these antibiotics.

Introduction

Strains of *Enterobacter cloacae* almost invariably produce a chromosomally-mediated β -lactamase, which has been designated 'Ia enzyme' by Richmond and Sykes (1973). About 80% of clinical isolates have inducible expression of this β -lactamase. A minority of isolates have stably-derepressed or basal expression of the Ia enzyme. Stably-derepressed organisms produce large amounts of β -lactamase without induction; basal organisms produce minimal amounts of the enzyme regardless of the presence of inducers. Stably-derepressed mutants tend to segregate from inducible strains at high frequency (Wiedemann, 1986).

In general, β -lactamase-inducible *E. cloacae* strains are susceptible to third-generation cephalo-

sporins and imipenem, but resistant to ampicillin and cefoxitin. Ureidopenicillins and carboxypenicillins are active against those β -lactamase-inducible isolates that lack plasmid-mediated β -lactamases such as TEM-1 enzyme. Stably-derepressed organisms are resistant to virtually all β -lactams, except imipenem and temocillin, and pose an increasing clinical problem (Sanders, 1983; Sanders and Sanders, 1985). β -Lactamase-basal strains are susceptible to most β -lactams, often including cefoxitin and ampicillin. Similar relationships between β -lactamase expression and antibiogram have been reported in laboratory mutants of *E. cloacae* (Curtis *et al.*, 1986).

In the present study we attempted to correlate antibiogram data for various inducibility types of *E. cloacae* with direct measurements of the β -lactamase-lability and inducer power of a range of newer β -lactam antibiotics.

Materials and methods

E. cloacae strains

β -Lactamase-inducible, β -lactamase-stably-derepressed and β -lactamase-basal strains were selected from a collection of 48 *E. cloacae* strains isolated from patients at the London Hospital during 1982–1983. Identification was by the API 20E system. Details of the selected organisms, and of mutants derived from them, are given in table I. Stably-derepressed mutants were selected from inducible strains by spreading $c. 5 \times 10^8$ cfu, from overnight cultures in Nutrient Broth No. 2 (Oxoid) on Diagnostic Sensitivity Test Agar (DST; Oxoid) containing cefotaxime 50 mg/L. The colonies that grew on this medium after overnight incubation at 37°C were subcultured on antibiotic-free DST agar, and then characterised. β -Lactamase-basal mutants were derived from stably-derepressed organisms by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Curtis *et al.*, 1978).

Antibiotics

Antibiotics were obtained as dry powders from suppliers as follows: ampicillin sodium and carbenicillin disodium from Beecham Research Laboratories, Brockham Park, Surrey; mezlocillin from Bayer Pharmaceuticals UK, Haywards Heath, Sussex; benzylpenicillin, cephaloridine and nitrocefin from Glaxo Group Research, Greenford, Middlesex; cefotaxime from Hoechst-Roussel, Somerville, NJ, USA; cefoxitin and imipenem from Merck, Sharp and Dohme Ltd, Hoddesdon, Herts, UK; ceftriaxone from Roche Products Ltd, Welwyn Garden City, Herts, UK; and cephalothin from the Sigma Chemical Co., St Louis, MO, USA.

Susceptibility tests

Minimum inhibitory concentrations (MICs) of antibiotics were measured in agar, with an inoculum of 10^4

cfu in 0.001 ml, taken from an overnight nutrient-broth culture.

β -Lactamase induction assays

The organisms were seeded into 10-ml volumes of Isosensitest Broth (Oxoid). After overnight incubation at 37°C, with continuous agitation, 1-ml volumes of these cultures were subcultured into pre-warmed 9-ml amounts of the same medium. Incubation was continued and inducers were added 90 min after subculture; 4 h after the addition of the inducers the cells were harvested by centrifugation at 5000 *g* and 37°C, then sonicated on ice. The β -lactamase activity of the sonicates was measured by spectrophotometric assay at 295 nm with 10 mM cephaloridine as the substrate in 0.1 M phosphate buffer, pH 7.0. The assay temperature was 37°C, and the light-path was 1 mm. Enzyme yields were standardised against protein concentration, which was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the reference standard.

β -Lactamase extraction

Cultures were grown overnight in Isosensitest Broth at 37°C, with continuous agitation, then diluted 10-fold into fresh identical broth, previously warmed to 37°C. Incubation was continued for 4 h, in the same conditions. Subsequently, the cells were harvested by centrifugation for 15 min at 5000 *g* and 37°C, washed once in 0.1 M phosphate buffer, pH 7.0, then resuspended at 150 times their original density in 0.1 M phosphate buffer, pH 7.0. β -Lactamase was released by subjecting the cell suspension to four cycles of alternate freezing and thawing. Debris was removed by centrifugation for 30 min at 100 000 *g* and 4°C, and the β -lactamase-containing supernates were stored at -20°C .

The β -lactamase extracted from 4 L of culture of *E. cloacae* strain 100 by this procedure was purified to

Table I. *E. cloacae* wild types and mutants and their β -lactamase production characteristics

Strain	Derivation	β -lactamase inducibility	Activity against cephaloridine†		
			not induced	induced with imipenem (1 mg/L)	induced with cefoxitin (100 mg/L)
100	Clinical isolate	SDR	31 800	20 100	32 800
100-def	NTG mutant of 100	B	22.1	25.8	56.8
84	Clinical isolate	SDR	16 100	38 200	40 600
84-def	NTG mutant of 84	B	56.8	497	345
684-I	Clinical isolate	I	45.8	20 700	5 800
684-con	Cefotaxime-selected mutant of 684I	B	25 100	34 900	44 700
684-def	NTG mutant of 684-con	B	42.2	40.8	43.2
144	Clinical isolate	B	62.2	101	72
158	Clinical isolate	B	9.10	14.4	7.2

B = basal; I = inducible; SDR = stably derepressed; NTG = N-methyl N'-nitro-N-nitrosoguanidine.

† Drug hydrolysed: nmol/min/mg of protein.

homogeneity by a two-step ion-exchange procedure (Livermore *et al.*, 1986). The mol. wt and purity of the enzyme were examined by SDS-PAGE, as described previously (Livermore *et al.*, 1986).

Hydrolysis and inhibition assays

Hydrolysis of β -lactams by crude and purified β -lactamase preparations was examined by UV spectrophotometric assay (O'Callaghan *et al.*, 1969; Waley, 1974). The antibiotic solutions were prepared in 0.1 M phosphate buffer, pH 7.0, and hydrolysis rates were measured at 37°C. The light path normally was 1 cm, but 1 mm light-path cuvettes were used for assays with high substrate concentrations (> 1 mM). The assay wavelengths selected for the antibiotics were as follows: ampicillin, benzylpenicillin, carbenicillin and mezlocillin 235 nm; cefotaxime 255 nm; ceftriaxone 257 nm; cefoxitin 260 nm; cephaloridine 255 nm and 295 nm; cephalothin 287 nm; and imipenem 297 nm. Kinetic parameters ($V_{\max}$, k_{cat} and K_m) were derived by linear regression analysis of Hanes (S/V vs S) plots of initial velocity (V) data obtained at 8–10 different substrate (S) concentrations.

Imipenem was tested as an inhibitor of cephaloridine hydrolysis under the following conditions: (i) where 1 mM cephaloridine and 0.1–200 nM imipenem were mixed in 1.1-ml total volumes of 0.1 M phosphate buffer, pH 7.0, and the reaction was started by addition of enzyme (25 μ l); or (ii) where 0.01–20 nM imipenem and enzyme were incubated together at 37°C for 15 min in 1.025 ml of 0.1 M phosphate buffer, pH 7.0, before addition of 100 μ l of 10 mM cephaloridine.

To isolate imipenem-inactivated β -lactamase, a mixture of imipenem (11.1 μ mol) and enzyme (1.1 nmol) was prepared in 0.3 ml of 0.1 M phosphate buffer, pH 7.0. After incubation for 15 min at 37°C the mixture was chromatographed at 4°C on a 30 $\times$ 0.9-cm column of Sephadex G25, which had previously been equilibrated in 0.1 M phosphate buffer, pH 7.0. Elution was with the same buffer at a flow rate of 0.25 ml/min and the time required for elution was determined during preliminary experiments with imipenem-untreated enzyme. The enzyme-containing fractions were collected, pooled, and warmed to 37°C. Samples were withdrawn periodically and their β -lactamase activity was compared to that of column-passaged imipenem-untreated enzyme, with 1 mM cephaloridine as the substrate.

Results

β -Lactamase production by *E. cloacae* wild type and mutant strains

Isoelectric focusing revealed that most (36 out of 48) of the *E. cloacae* isolates from clinical specimens produced a form of Ia β -lactamase that focused at pI 8.4. Relative hydrolysis rates of 1 mM cephaloridine, 1 mM cephalothin and 1 mM benzylpenicillin

by this β -lactamase were in the approximate ratios 100:57:3, regardless of the strain used as a source of enzyme.

Strains with inducible, stably-derepressed and basal pI 8.4-enzyme expression were selected for detailed study, and quantitative induction data for these organisms, and their laboratory-derived mutants, are given in table I. Strain 684I had inducible expression of the enzyme, but derepressed mutants segregated at an approximate frequency of 10^{-8} . Wild-type strains 84 and 100, and the laboratory-derived mutant 684-con, constitutively produced large amounts of the β -lactamase, whereas the basal mutants 684-def and 100-def and the isolates 144 and 158 had only trace amounts of the enzyme. Mutant 84-def produced more β -lactamase than the other β -lactamase-basal organisms, but much less than its stably-derepressed parent strain, 84.

None of the strains studied in detail produced plasmid-mediated-type β -lactamases. Such enzymes, however, were present in 20 of the 48 clinical isolates.

Antibiotic susceptibility

The β -lactamase-inducible strain 684I was very susceptible (MIC < 4 mg/L) to all the β -lactams tested except ampicillin and cefoxitin (table II). Its stably-derepressed mutant, 684-con, was highly resistant (MIC > 128 mg/L) to all the β -lactams except imipenem, as were the stably-derepressed clinical isolates 84 and 100. Strains 684I and 684-con were both equally susceptible to imipenem (MIC = 0.25 mg/L).

The β -lactamase-basal isolates 144 and 158, and laboratory mutants 100-def and 684-def, were susceptible to 16 mg/L or less of all the β -lactams tested, including ampicillin and cefoxitin. Mutant 84-def, which produced larger amounts of Ia enzyme than the other basal mutants, remained resistant to ampicillin and cefoxitin (MIC 64–512 mg/L) and was only moderately susceptible to third-generation cephalosporins and mezlocillin (MICs 8–16 mg/L).

Mutant 684-def was only 2–4-fold more susceptible than strain 684I to third-generation cephalosporins, mezlocillin and carbenicillin, but was four-fold more susceptible to imipenem and > 128-fold more susceptible to ampicillin and cefoxitin.

MICs of non- β -lactam antibiotics (gentamicin, chloramphenicol, tetracycline and nalidixic acid) remained constant within each mutant series (data not shown), suggesting that permeability changes had not arisen simultaneously with altered β -lactamase synthesis.

Table II. Susceptibility of *E. cloacae* wild type and mutant strains to β -lactam antibiotics

Strain	MIC (mg/L)						
	ampicillin	carbenicillin	mezlocillin	cefotaxime	ceftriaxone	cefoxitin	imipenem
100	2084	64	128	512	512	1024	0.5
100-def	4	16	2	0.12	0.12	4	0.06
84	> 2048	128	128	256	512	256	1
84-def	512	16	8	8	16	64	0.25
684I	512	4	4	0.5	0.25	256	0.25
684-con	2048	256	128	256	256	512	0.25
684-def	4	2	1	0.06	0.06	2	0.06
144	16	2	2	0.25	0.5	8	0.25
158	1	2	2	0.015	0.12	4	0.25

Inducer power of β lactams

The inducer power of β -lactams was examined for strain 684I, and the results are summarised in fig. 1. Imipenem appeared to be the strongest inducer at low concentrations, giving 200–300-fold induction when used below 1 mg/L. Cefoxitin also was a strong inducer, giving 100-fold induction at 10 mg/L. The other β -lactams were very poor inducers below 10 mg/L, but caused up to 40-fold induction at 100 mg/L.

The values of induction ratios should be taken as only semi-quantitative because their calculation is subject to several distortions. In particular, (i) the assays measured only cell-bound β -lactamase and significant enzyme leakage may have occurred, especially from cells exposed to supra-MIC antibiotic concentrations, (ii) the induction is time dependent and the choice of induction period was arbitrary and (iii) residual inducer may interfere with β -lactamase assay. Nonetheless there was a dramatic difference between imipenem and cefoxitin, which induced strongly below its MIC value, and cefotaxime, ceftriaxone, mezlocillin and carbenicillin which failed to do so.

Fig. 1. β -Lactamase induction in *E. cloacae* strain 684I by imipenem $\circ$ — $\circ$, cefoxitin $\bullet$ — $\bullet$, cefotaxime Δ — Δ , ceftriaxone $\blacktriangle$ — $\blacktriangle$, carbenicillin $\triangle$ — $\triangle$, ampicillin $\blacksquare$ — $\blacksquare$, and mezlocillin $\square$ — $\square$. Induction ratios were calculated as the enzyme quantity/mg of cell protein in the presence of inducer, divided by enzyme quantity/mg of cell protein in the absence of inducer.

Hydrolysis of β -lactams by β -lactamase from *E. cloacae* strain 100

E. cloacae strain 100 was selected as a source of the pI 8.4 enzyme for detailed hydrolysis studies. The enzyme preparation purified from this organism contained a single protein species, which had a mol. wt of 46 000, as measured by SDS-PAGE. Kinetic parameters for the hydrolysis of β -lactams by this enzyme are listed in table III. The k_{cat} value (molecules of antibiotic hydrolysed per molecule of enzyme per unit time) for cephaloridine was 45 times that for benzylpenicillin; however the K_m of

cephaloridine (610 μ M) was much higher than that of benzylpenicillin (10 μ M), with the result that k_{cat}/K_m ratios were similar for the two antibiotics. For cefoxitin and ceftriaxone, k_{cat} values were in the range 0.2–1/min; those for ampicillin, carbenicillin, mezlocillin and cefotaxime were in the range 5–40/min. K_m values for carbenicillin, cefoxitin and ceftriaxone were below 2 μ M; those for ampicillin, mezlocillin and cefotaxime were in the range 5–44 μ M. We did not observe non-Michaelis-Menten hydrolysis kinetics for any of the agents tested,

despite using antibiotic concentrations as low as $1.25 \mu\text{M}$.

Hydrolysis of imipenem was too slow to be measured accurately; however, the compound did inhibit the enzyme, and the kinetics of this behaviour were investigated. The I_{50} concentration of imipenem for inhibition of the hydrolysis of 1 mM cephaloridine was $0.19 \mu\text{M}$ when the enzyme and imipenem were not pre-incubated, but was decreased to $0.04 \mu\text{M}$ when the enzyme and imipenem were incubated together for 15 min at 37°C before adding of cephaloridine. These results suggested the accumulation of an enzyme-imipenem complex during the pre-incubation period, and isolation of this complex by gel-filtration chromatography was undertaken. The conditions were such that free, or imipenem-inactivated, β -lactamase eluted with the void volume of the column, about 30 min after loading, and was separated completely from unbound imipenem, which was retained in the gel matrix for $4\text{--}5 \text{ h}$. Immediately after elution, the imipenem-treated enzyme lacked catalytic activity against 1 mM cephaloridine. However, the enzyme activity recovered progressively when the complex was incubated at 37°C . This recovery obeyed first-order kinetics, such that a plot of log percentage inactivation of the enzyme *versus* time was linear (fig. 2). The half-life of the inactivated species was about $47 \pm 2 \text{ min}$.

Discussion

Hyperproduction of Ia β -lactamase by *E. cloacae* has been associated with the development of resistance to most new β -lactams except imipenem (Sanders and Sanders, 1985). Hyperproduction

Table III. Kinetics of hydrolysis of β -lactams by *pI* 8.4 β lactamase from *E. cloacae* strain 100

Antibiotic	$k_{\text{cat}}(\text{min}^{-1})^*$	$K_m(\mu\text{M})$	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)
Benzylpenicillin	488	10	48.8
Cephaloridine	22 000	610	36.1
Cefotaxime	40	44	0.91
Ceftriaxone	0.82	1	0.82
Cefoxitin	0.24	1.5	0.16
Ampicillin	55	4	13.75
Carbenicillin	5.24	0.15	34.9
Mezlocillin	9.81	5.5	1.98

* Maximum number of antibiotic molecules hydrolysed per minute by one enzyme molecule.

Fig. 2. Recovery of cephaloridine-hydrolysing activity by the isolated *pI* 8.4 enzyme-imipenem complex. The complex was incubated at 37°C and samples were periodically withdrawn and tested (see *Methods*). Percentage inactivation was calculated as $[(A_0 - A)/A_0] \times 100$ where A_0 was the activity of column-passaged, imipenem-untreated-enzyme and A was the activity of column-passaged imipenem-treated enzyme.

arises transiently in inducible strains during exposure to strong inducers, and permanently in stably-derepressed mutants, which have an altered β -lactamase-regulatory gene (Lindberg and Normark, 1986). Stably-derepressed mutants are segregated by inducible populations at high frequency (10^{-5} – 10^{-8}) and may be selected during clinical exposure to β -lactams (Sanders, 1983). Similar induction and selection phenomena are observed in other Class I β -lactamase-inducible species, such as *Pseudomonas aeruginosa*, *Citrobacter* spp., *Serratia* spp. and the indole-positive *Proteus* spp. (Sanders and Sanders, 1985; Livermore, in press). In this study we attempted to relate MIC data for β -lactamase inducible, stably-derepressed and basal *E. cloacae* isolates and mutants to direct measurement of the β -lactamase lability and inducer power of various β -lactams. All the strains examined produced a *pI* 8.4 form of the Ia enzyme, which we have found to be the commonest subtype of Ia enzyme in *E. cloacae* isolated at the London Hospital.

MICs of all the β -lactams for the stably-derepressed strains and mutants exceeded those for the basal strains and mutants, indicating that hyperproduction of the *pI* 8.4 enzyme was protective against all these agents including imipenem. However, the protection against imipenem was much

less than against the penicillins and cephalosporins that we tested. The MICs of imipenem for the derepressed isolates and mutants, unlike those of other β -lactams, remained in the therapeutic range.

For all the compounds except imipenem, the protection afforded to the derepressed organisms by the *pI* 8.4 β -lactamase could be correlated with the hydrolytic activity of this enzyme. Although the k_{cat} values of the enzyme for cefoxitin, ceftriaxone, mezlocillin and carbenicillin were $<0.1\%$ k_{cat} cephaloridine, suggesting high stability, the K_m values of these antibiotics also were low, giving high k_{cat}/K_m ratios. As has been discussed elsewhere, the magnitude of the k_{cat}/K_m ratio, ($= V_{\text{max}}/K_m$ divided by enzyme quantity) is the critical determinant of the hydrolytic efficiency of a β -lactamase against the low antibiotic concentrations that may be attainable in the bacterial cell (Livermore, 1983, 1985; Vu and Nikaido, 1985; Bush and Sykes, 1986). A high k_{cat}/K_m ratio suggests that the enzyme may be efficient against the antibiotic inside the cell. Unlike the other compounds tested, imipenem was not hydrolysed at a significant rate by the *pI* 8.4 β -lactamase; however, it did inhibit the activity of this enzyme against cephaloridine. The inhibition was increased when imipenem and enzyme were pre-incubated before addition of the substrate, suggesting the accumulation of an inert imipenem- β -lactamase complex. Gel filtration allowed the isolation of this complex, which was found to have a breakdown half-life of 47 ± 2 min. Since this half-life exceeds the generation time for exponentially growing *E. cloacae* (*c.* 20 min) it seems likely that the feeble protection that the β -lactamase provided against imipenem depended, at least partly, on a non-hydrolytic mechanism, with the enzyme trapping the imipenem molecules. We have no direct data on the chemical nature of the imipenem-Ia enzyme complex; however, similarly stable Class I β -lactamase-antibiotic complexes commonly contain a covalent acyl link between the enzyme and the β -lactam and are normal intermediates on a hydrolytic pathway (Bush *et al.*, 1982). Theoretical considerations suggest that such covalent trapping is a credible cause of β -lactamase mediated resistance (Livermore, 1987) whereas the non-covalent trapping suggested previously remains arguable (Livermore, 1985, 1987).

Inducible expression of the *pI* 8.4 enzyme conferred narrower-spectrum resistance than did stable derepression. Thus, although MICs of imipenem, ampicillin and cefoxitin for strain 684I were similar to those for 684-con, those of cefotaxime, ceftriaxone, mezlocillin and carbenicillin

remained almost as low as for 684-def and the other β -lactamase-basal organisms. We could relate these findings to the direct observation that the cephalosporins, mezlocillin and carbenicillin were very weak β -lactamase inducers at low concentrations (fig. 1). Thus, although labile, it seems that these compounds inhibit growth at concentrations below those required to induce protective quantities of the *pI* 8.4 β -lactamase in inducible strains such as 684I. Conversely imipenem, cefoxitin and, to a lesser extent, ampicillin were strong inducers below their MIC values, with the result that inducible β -lactamase expression was almost as protective as stably-derepressed expression.

The combination of lability with weak inducer power explains the tendency for cephalosporins and ureidopenicillins to select stably-derepressed mutants from β -lactamase-inducible *E. cloacae* populations. Numerous cases have been reported where selection of this type has occurred in patients receiving therapy with labile weak inducers (Sanders and Sanders, 1985; Livermore, 1986, and in press). Selection of derepressed mutants seems unlikely with imipenem, since it has equal activity against β -lactamase inducible and stably-derepressed organisms (table II, also Goering *et al.*, 1982). This prediction is supported by the results of Kirkpatrick *et al.* (1986), who were unable to select β -lactamase-derepressed *E. cloacae* with imipenem.

The present results for *E. cloacae* broadly resemble previous data for *P. aeruginosa* (Livermore, 1986; Livermore and Yang, 1987). Cefotaxime, ceftriaxone and ureidopenicillins were labile weak inducers for the Class I enzymes of both species, whereas imipenem was a strong inducer that retained activity against inducible and stably-derepressed organisms, despite marginal β -lactamase lability. However the behaviour of carbenicillin differed markedly between the two species: for *E. cloacae* carbenicillin was a labile weak inducer, behaving similarly to mezlocillin, whereas it is stable to the *P. aeruginosa* enzyme and retained almost equal activity against β -lactamase-inducible, β -lactamase-stably-derepressed and β -lactamase basal organisms (Livermore and Yang, 1987).

Several minor differences were apparent between the *pI* 8.4 sub-type *E. cloacae* Ia enzyme, studied here, and the more widely-studied, but rarer, P99 sub-type. In particular, the *pI* 8.4 enzyme exhibited moderate k_{cat} (40/min) and K_m (40 μM) values for cefotaxime, whereas k_{cat} and K_m of this drug for P99 enzyme are about 1/min and 1 μM respectively (Bush *et al.*, 1982; Livermore *et al.*, 1986). The *pI* 8.4 enzyme also differed from the E-2 form of Ia enzyme described by Seeberg *et al.* (1983) and Bush

et al. (1985), both in its kinetics of activity against cefotaxime, wherein E-2 resembled P99 enzyme, and in the stability of its imipenem complex. Bush *et al.* (1985) reported a half-life of only 0.8 min for the E-2 enzyme-imipenem complex; considerably less than that observed here for the pI 8.4 enzyme. However, this diversity of Ia β -lactamase types in

E. cloacae seems largely of academic interest since the stably-derepressed strains are reported to show a similar spectrum of resistance, irrespective of the enzyme-sub-type that they produce.

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